Introduction

1.1 Global malaria scenario:

Malaria is one of the major killer diseases responsible for about one million deaths every year around the world. It is one of the most important infectious diseases in the tropical and the sub-tropical regions of the planet earth. At present, about 100 countries or territories are considered malarious, with nearly 50% of them in Sub-Saharan Africa (Figure 1). Other malaria endemic areas mostly belong to Southeast Asia and Latin America. Globally, approximately 3 billion people corresponding to 40% of the world’s population are at risk of infection (Hay et al., 2004). The South East Asian countries reports >100 million cases (WHO, 2010), whereas Indian records about 2 million cases annually (NVBDCP, 2010). Among the four malaria parasite species, *P. falciparum* has been reported to contribute about 50% of the total cases.

Fig1: Malaria map of the world
Malaria is a vector-borne parasitic disease caused by intracellular protozoan parasites of the genus *Plasmodium*. Four species, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, infect humans. Another malaria species *Plasmodium knowlesi* which is macaque malaria parasite also affect human and its prevalence is increasing in some Southeast Asian countries. The parasites multiply asexually in the human host and go through sexual reproduction in the female anopheline mosquito vector (Figure: 2). Each type of infection causes debilitating febrile illness, but approximately 90% of clinically manifest infections are caused by *P. falciparum* while *P. vivax* accounts for nearly 10% of the global malaria incidence. The main causes of mortality are severe anemia and the cerebral malaria caused by *P. falciparum*. Recent estimates suggest that between 500 million and 5 billion clinical episodes and up to 3 million deaths occur each year due to malaria, with Sub-Saharan Africa having 90% of this mortality burden. Moreover, the devastating consequences of malaria are a major obstacle to social and economic development in affected regions (Breman et al., 2004; Mendis et al., 2001; Snow et al., 2005).

*Figure 2: Malaria Parasite Life cycle*
1.2 Malaria situation in northeastern India

Northeastern part of India which comprises of eight states is a very strategically important area. These states shares long International border with countries like Bhutan, China, Myanmar and Bangladesh and also interstate border among themselves. All the international borders are mostly hills and foothills covered by thick forest cover and lack proper communication and health infrastructure. Due to congenial climate, difficult terrain, unstable population, human migration and other malariogenic factors these states are highly endemic to malaria (Mahanta et al., 1998). Although the population of north eastern region is only 4% of the country, but records about 10% of total malaria cases in India annually. *P. falciparum* is the major infection throughout these states causing considerable mortality. About 11% of the *P. falciparum* cases of India reported from these states (Mahanta et al., 1998). Among all the Northeastern states Assam is the most populated state and contribute majority of the malaria cases (~ 50%) followed by Arunachal Pradesh, Meghalaya and Tripura (NVBDCP data). Malaria cases are reported across the region, there is greater concentration of cases in the foothill areas and places which lie close to interstate/international borders (Dhiman et al., 2010a; Dhiman et al., 2011). As the region has uneven malaria distribution and frequent localized focal out breaks, controlling has been a daunting task.

1.3 Malaria Control:

Malaria control program mostly rely on two aspects viz- mosquito vector control and parasite control in human host. Both control strategies are integral part of control program and its success depends largely on effective implementation of these two components. In recent time malaria control program all over the world have faced tremendous pressure from insecticide resistance in mosquito vectors and drug resistance
in parasite. Most of the commonly used insecticides are becoming ineffective against vector mosquitoes to varying degree. Moreover, mosquitoes are also changing their behavior to avoid insecticide and there by escape itself from its killing effect. Therefore new insecticide and different delivery systems are required for mosquito vector control.

1.4 Chemotherapy against Malaria:

Chemotherapy against the malaria parasite is an integral part of every malaria control program. Presently no effective vaccine is available against the malaria parasite and as such chemotherapy holds a significant position in malaria control.

Treatment of malaria came a long way since ancient times from herbal products to synthetic antimalarial drugs. Many of the current synthetic drugs were also derivatives of the herbal molecules those have in use since ages. Currently available antimalarial compounds belong to three distinct groups viz- aryl amino alcohol compounds (chloroquine, quine, mefloquine, amodiaquine, halofentrine, lumefentrine, primaquine); the antifolates (pyrimethamine, trimethoprim, proguanil) and artemisinin derivatives (artemisinin, dihydroartemisinin, artesunate, arteether).

Chloroquine and other Quinoline like Mefloquine and Primaquine are the most widely used drugs so for the treatment of malaria. Due to high efficacy and low toxicity chloroquine has been the drug of choice for treatment of malaria in many parts of the world including India.

1.5 Antimalarial Drug resistance:

One of the greatest challenges faced by malaria control program worldwide, is the anti malarial drug resistance. Spread of malaria to new areas or re-emergence of malaria in
areas where the disease has been eradicated could be attributed to antimalarial drug resistance. Human migration, population movement due to development or natural disaster etc fueled the spread of drug resistance to new frontiers. Drug resistance has also played a significant role in the occurrence and severity of epidemics in some parts of the world (Bloland et al., 2001).

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bruce-Chwatt et al., 1986). This definition of drug resistance requires demonstration of parasitemia in blood after receiving complete dose of antimalarial drug and also adequate plasma concentration drug or its metabolite. However, in most of the in vivo studies for commonly used antimalarials such as CQ drug metabolism study may not be necessary as patient receiving directly observed therapy is usually considered sufficient (Slutsker et al., 1990).

Out of the four species of malaria that naturally infect human, P. falciparum and P. vivax has developed resistance to antimalarials, although the geographical distribution of resistance to single antimalarial drug varies greatly (Parija et al. 2011). Resistance to all classes of antimalarials has been developed across the malaria endemic zones of the whole world (Figure: 3) except Artimisinin group compounds. Chloroquine (CQ)-resistant strains of P. falciparum first appeared in the late 1950s, almost simultaneously in Southeast Asia and South America (Spencer et al., 1985; Wernsdorfer et al., 1991; Young et al., 1961), and subsequently spread through most of the regions where P.
*P. falciparum* is endemic. Sulfadoxine pyrimethamine (SP) was next used as the drug of choice against CQ-resistant malaria; however, resistance quickly emerged on the Thai-Cambodian border around 1980 and is now found throughout most of the Southeast Asia, the Amazonian basin of South America, and Africa (Ahmed *et al.*, 2004; Bjorkman *et al.*, 1990; Ronn *et al.*, 1996). However, there are few reports of resistance to Artemisinin derivatives from some areas (White *et al.*, 2009; Talisuna *et al.*, 2004). Antimalarial resistance to *P. vivax* has emerged comparatively later and is seen mostly in Southeast Asia (Achan *et al.*, 2011; Talisuna *et al.*, 2004). In India resistance to CQ has been first reported from Assam by Sehgal *et al.* (1973). Resistance to SP combination therapy was first reported from Delhi in 1987 (Choudhury *et al.*, 1987) and later on from other parts of India including Northeastern India. Quinine resistance was first observed in South America nearly a century ago. In 1960s this was also reported from Thai Cambodian border and other parts of Southeast Asia. However quinine resistance is not wide spread as CQ. In India quinine resistance was reported from the Northeastern States and Karnataka (Mishra., 1996). Resistance against another important antimalarial mefloquine was also reported from Thai Cambodian Border in 1980s (Wongsrichanalai *et al.*, 2002).

Emerging trend of antimalarial resistance giving control programmes a serious challenge as very few drugs available in the armory to combat malaria. Maybe due to increased selection pressure and indiscriminate use resistance to these drugs emerged in different places across the globe. For effective malaria management knowledge of drug susceptibility is vital, otherwise disease can become uncontrollable and become more complicated which will have long term consequences.
1.6 Molecular Epidemiology:

Recent development in molecular biology techniques have contributed tremendously in building genomic knowledge base of many organisms. Malaria parasite is one of the most elaborately studied organisms in all aspects. Parasites are haploid for most of their life cycle and have a short duration of diploid life inside mosquito vector. Each parasite genome consists of 23 megabases (Mb) distributed over 14 chromosomes, with separate mitochondrial and plastid genomes of 6 and 35 kilobases, respectively. The genomic information of malaria parasite can lead to solutions of many unanswered questions. Molecular epidemiology can be defined as the “study of pathogen genotypes and gene expression as it relates to the occurrence of infection and disease in human populations”. In one sense, this is just an extension of normal epidemiological analysis to incorporate any molecular information on pathogens detected within individuals. Disease epidemiology is a very vital subject in understanding of the disease and its
control. Disease dynamics, its spread, clinical profile population affected etc. are the basic epidemiological studies which are must for controlling of the disease. With advances in the molecular biology techniques, different genomic data of the parasite were also become available which aided in understanding of the disease and its epidemiology. Thus the molecular epidemiology is an epidemiological study in the light of genomic information. The goal of molecular epidemiology is to see beyond a description of variability to detect the effects of molecular and cellular processes in infection, pathogenesis, immunity, or therapeutic responses.

*Plasmodium falciparum* exhibits great diversity in ecological and epidemiological characteristics and also extensive polymorphism in genes encoding antigenic proteins and mutations in certain genes responsible for resistance to antimalarial drugs. Investigation of genetic polymorphism of *P. falciparum* is of prime importance for better understanding of the population genetic structures and dynamics of local parasite population. The outcome of an infection depends upon a complex matrix of interrelated factors *i.e.* the parasite, the host’s innate immunity and acquired immune response and also on environment. In case of *P. falciparum* the situation become further complicated both with respect to antigenic diversity and sexual reproduction of the parasite. Due to this it can potentially generate novel chromosome assortment, gene combination and allele from heterozygous oocyst, which is worrying in the long run, because it is able to generate endless source of novelty.

Monitoring of antimalarial resistance is mainly done by *in vivo* test as per WHO guidelines. Though this is an expensive time consuming and specialized study which requires much expertise yet gives better results from treatment point of view. Alternatively *in vitro* culture method can also be used to determine the drug resistance.
This is also an expensive method and requires good laboratory facilities but not always practically feasible as the parasite does not adapt to the *in vitro* culture (Sharma, 2012). Therefore, attempts were made to develop molecular markers which can give idea about drug resistance status of the parasite population.

Chloroquine resistance in *P. falciparum* is mediated by a parasite food vacuole membrane transporter molecule, encoded by the gene *pfcr* present on chromosome 7 of the parasite. Mutations at several amino acid positions of this protein have been reported in resistant strains. Mutation at 76 position where lysine is replaced by threonine is mostly present in all the resistant cases and therefore it was mostly came to use in *in vitro* Chloroquine resistance monitoring (Fidock *et al.*, 2000, Sidhu *et al.*, 2002 and Wellems *et al.*, 1991). Multiple studies in *falciparum* malaria endemic areas have suggested that K76T mutant parasites are linked to chloroquine resistance in both *in vitro* and *in vivo* trials (Djimde *et al.*, 2001a; Vinayak *et al.*, 2003). The K76T mutation has also been found associated with amodiaquine resistance and predictive treatment failure for both chloroquine and amodiaquine (Beshir *et al.*, 2010 Folarin *et al.*, 2011; Picot *et al.*, 2009). Three other mutation in the adjacent upstream of 76th position i.e. 72nd 74th and 75th position with the monomorphic 73rd position form different *pfcr* haplotype. Two major haplotypes CVIET and SVMNT are mostly found to be associated with Chloroquine resistance in malaria endemic regions (Awasthi *et al.*, 2011; Vathsala *et al.*, 2004). These data can provide valuable information of spread and evolutionary history of chloroquine resistance in *P. falciparum*.

Another gene *Plasmodium falciparum* multi drug resistance gene (*Pfmdrl*), which is localized on chromosome 5, encodes a P-glycoprotein homolog (Pgh1) and has been localized to the parasite DV (Foote *et al.*, 1989; Cowman *et al.*, 1988) and thought to
play role in conferring CQ resistance in *P. falciparum*. Pgh1 has a typical structure shared by members of the ATP binding cassette (ABC) transporter family (Endicott and Ling, 1989). Sequence analysis of the gene revealed its dimorphic structure and association of a phenotype with CQ resistance. However, its role in conferring resistance has been doubted but can be correlated with increase in resistance level (Basco and Ringwald, 2002; Reed *et al.*, 2000). In several field epidemiological studies it has been found that association of *pfmdr1* N86Y mutation with CQ resistance (Basco *et al.*, 1995; Nagesha *et al.*, 2001; von Seidlein *et al.*, 1997).

Molecular monitoring of drug resistance is proved to be a handy tool as it is less expensive, short and devoid of complications which may arise during *in vivo* drug sensitivity. However, its effectiveness has to be ascertained in different areas and different epidemiological conditions. Studies on molecular epidemiology of drug resistance are very limited in Northeastern states, only a few studies have been carried out so far. Therefore, it is pertinent to study molecule epidemiology of drug resistance for formulation and implementation of drug policy.

The genetic complexity of *Plasmodium falciparum*, the infectious agent of malignant malaria, and in particular its ability to generate mutant variants, make it a successful pathogen. Genetic variants are involved in pathogenicity and in immune responses and have led to the emergence of resistance against virtually any drug available for treatment. Such variants are under strong selective pressure.

*P. falciparum* also exhibits high degree of genetic polymorphism in the gene encoding antigenic proteins. Most of the surface antigens such as MSP 1, MSP 2, GLURP which contain peptide repeats, show length polymorphism. These proteins are immunogenic and the host produces a significant amount of antibodies against it. In most of the cases,
it has been found that this could be a mechanism adopted by the parasite to keep the host’s immune system preoccupied, so that it can escape hosts immune attack and survive in the hostile host and prolong the duration of infection. Antigenic variation and genetic polymorphism had posed great difficulty in raising a universal malaria vaccine. Antigenic diversity of malaria parasite can be correlated to different parameters such as transmission intensity, severity of the disease, complexity of infection, host immune response etc. Transmission intensity of malaria in a given area is a very important parameter in context of control strategy. Complexity of an infection i.e. simultaneous infection with different parasite or different strain create hurdle in the treatment of the disease.

The merozoite surface proteins MSP-1 (Kimura et al., 1990) and MSP-2 (Fenton et al., 1991) with numerous alleles and differing in the length of the genes have been extensively studied and their genetic polymorphisms were used to describe clonality of infections in a large number of studies. Length variability mainly results from repeat sequences. MSP-1 can be divided into 17 blocks and the block 2 is of particular importance (Tanabe et al., 1987) since it undergoes rapid intragenic recombination and play significant role in acquired immunity of the host (Conway et al., 2000). Distinct MSP alleles were associated with distinct HLA class II alleles, points also to an immune selection of MSP variants by the human host (May et al., 1999). MSP-2 with the allele groups FC27 and 3D7 has been considered as the most informative marker of clonality and genotyped in many studies to assess the degree of multiple infections. It also play important role in host’s acquired immunity. Variability of the glutamine-rich protein (GLURP) (Borre et al., 1991), exhibited by length polymorphism, has also been described and determined in studies on multiclonal P. falciparum infections.
Studies on genetic diversity of the *P.falciparum* in different endemic locations of the Northeastern India will help in understanding the infection complexity, role of transmission intensity in genetic diversity etc. Due to various factors, only a few studies in this regard have been carried out in this region. Hence, this study will be able to contribute humbly towards the understanding of *P. falciparum* drug resistance, role of SNPs in conferring drug resistance and also genetic diversity.
Aims and Objectives

1. *In vivo* drug sensitivity trial to determine anti malarial drug resistance status in different malaria endemic areas.

2. To study the different molecular markers in conferring drug resistance to *Plasmodium falciparum*

3. Studies on genetic diversity of *Plasmodium falciparum* in different malaria endemic areas and its relation to disease severity and transmission intensity.